Catechin, caffeine and theobromine are three bioactive compounds that are present in plant foods and are major constituent of tea, coffee and cocoa drinks respectively. Although not structurally related, catechin, caffeine and theobromine have been reported to elicit psychostimulatory properties. In this study, we investigated the antioxidant properties of the compounds through their radicals [1,1-phenylendiamin (DPPH), 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonate (ABTS), nitric oxide (NO), and hydroxyl (OH)] scavenging abilities, ferric reducing potentials and Fe2+ chelating abilities. The effect of the compounds on different prooxidants (FeSO4, cistplatin and sodium nitroprusside) induced lipid peroxidation in rat brain homogenate was assessed. Also, the effects of the compounds on some cholinergic [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] enzymes in rats brain homogenates were assessed. The results revealed that caffeine, catechin and theobromine had antioxidant properties and exhibited inhibitory effects on activities of AChE and BChE. Catechin had the best antioxidant property while theobromine produced the highest enzyme inhibition effect. These findings may provide new insight into the effects of these bioactive compounds as obtained in many foods especially with respect to their antioxidant and neuroprotective effects.

Keywords: Antioxidant, Psychostimulatory, Caffeine, Theobromine, Catechin

INTRODUCTION

Enteric tea, coffee and cocoa (Theobroma cacao L.) appear to be part of the most promising plant-based foods due to their high polyphenol (majorly catechin) and methylxanthines: caffeine (the most active component) and theobromine contents evidently highlight the link with their health-promoting properties (Mitchell et al., 2004; Andújar et al., 2012; Yan et al., 2020). Tea is consumed by over two-thirds of the world’s population making it the most popular beverage in the world (Ide et al., 2018). Many studies have shown positive effects of green tea (Camellia sinensis) and tea-based products due to their contents of catechin and its derivatives (Li et al., 2018). Catechins are flavanols which belong to polyphenol compounds that have an important role in protection against degenerative diseases (Ide et al., 2018). Catechin has ability to decrease neuronal damage by reducing oxidative stress, scavenging reactive oxygen species and improving antioxidant enzymes (Xiang et al., 2016). Caffeine has been the subject of extensive research for its occurrence in nature and its long history of use (Kolayli et al., 2004). It is a major alkaloid found in coffee, cocoa, tea, cola drinks, chocolate and other plant-based foods. Research has shown that caffeine is a pharmacologically active substance (Almeida et al., 2006), a mild central nervous system stimulant (Svílaas et al., 2004) with potential cognitive enhancement (Abreu et al., 2011) and antioxidant properties (Noschang et al., 2009) among others. Caffeine, a methylxanthine, is one of the most popular and commonly consumed drugs in the world (Kolayli et al., 2004). On the other hand, theobromine (3,7-dimethylxanthine) is the product of...
caffeine metabolism in bacteria and plants (Dash and Gummadi, 2006). Theobromine is found naturally in cacao beans (Gummadi and Devarai 2006) and is the source of the typically bitter taste of chocolate. Theobromine has a milder effect than caffeine. It acts as a vasodilator and has a moderate effect as a chemical signaling molecule (Mumford et al., 1994). Theobromine is characterized as an antioxidant and prooxidant (Sonish et al., 2005). Free radicals generated within the brain due to oxidative stress are the major factor in Alzheimer’s disease (Hertog et al., 1992). These radicals cause a permanent irreversible oxidative damage to brain cells, which further aggregates to loss of memory or dementia (Valko et al., 2007). Antioxidants are substances that when present at low concentrations, compared with those of the oxidizable substrate, significantly delay or inhibit oxidation of that substrate (Gomez-Ruiz et al., 2008). Researchers have shown that the intake of cereals, fruits, vegetables, tea, and coffee are important to lower risk of diseases that are formed as consequence of free radicals (Dorea and da Costa 2005; Podsedek 2007). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are key enzymes of the cholinergic system that play critical role in the pathogenesis of neurodegenerative diseases especially Alzheimer’s disease (AD) (Lendvai and Vizi, 2008). Impairment of the cholinergic neuron has been implicated in the pathogenesis of AD and major therapeutic strategy for managing this disease involves the use of cholinesterase inhibitors (Lendvai and Vizi, 2008). Based on the plausible information, it is thus justifiable to attempt to determine the most active neurostimulant amongst the three beverages. The aim of our study was to compare and rate the effects of caffeine, catechin and theobromine on antioxidant activity.

We measured the antioxidant properties of the compounds through their radicals [1,1 diphenyl-2-pirclylhydrazyl (DPPH), 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS), nitric oxide (NO), and hydroxyl (OH)] scavenging abilities, ferric reducing potentials and Fe²⁺ chelating abilities. In addition, the effects of the compounds on different prooxidants (FeSO₄, cisplatin and sodium nitroprusside) induced lipid peroxidation in rat brain homogenates were assessed. We also measured their cholinergic [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] and monoaminergic [monoamine oxidase (MAO)] activities in rat brain.

**MATERIALS AND METHODS**

**Reagents**

Catechin, caffeine, theobromine, reduced β-Nicotinamide adenine dinucleotide 2-phosphate (NADPH), thiobarbituric acid (TBA), deoxyribose, Folin–Ciocalteau’s reagent, acetylthiocholine iodide, butyrylthiocholine iodide, were procured from Sigma-Aldrich, Inc., (St Louis, MO, USA). Hydrogen peroxide, methanol, acetic acid, thiourea, copper sulphate, sulphuric acid, sodium carbonate, aluminum chloride, potassium acetate, sodium dodecyl sulphate, iron (II) sulfate, potassium ferricyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England).

**Sample preparation**

The stock concentration of catechin, caffeine and theobromine (1 mg/ml) were prepared according to the method of Kantamala et al. (1990) and then kept at -4°C for subsequent analysis.

**Determination of 2,2-diphenyl - 1- picrylhydrazyl (DPPH) radical scavenging ability**

The free radical scavenging ability of the bioactive compounds against DPPH free radical was assessed as earlier described (Gyamfi et al., 1999). 1mL each of the diluted bioactive compounds at different concentrations (40- 160 µg/mL) were mixed with 1mL 0.4mM DPPH radicals dissolved in methanol. The mixture was left in the dark for 50 min, and the optical density was taken at 516nm. 2mL DPPH solution without the test samples was used as the control. The DPPH free radical scavenging ability was then calculated as percentage of the control. All assays were performed in triplicate.

**Determination of total antioxidant capacity**

The total antioxidant capacity was evaluated based on 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate radical (ABTS⁺) scavenging ability of the bioactive compounds (Re et al., 1999). Using trolox as the standard, the trolox equivalent antioxidant capacity (TEAC) was calculated.

**Fenton reaction (Inhibition of degradation of deoxyribose)**

The ability of the bioactive compounds to prevent Fe²⁺/H₂O₂-induced decomposition of deoxyribose was carried out using the method (Halliwell et al., 1981). The percentage (%) OH radical scavenging ability was calculated.

**Determination of ferric reducing antioxidant property (FRAP)**

The ferric reducing antioxidant property of the bioactive compounds was determined by assessing the ability of the bioactive compounds to reduce FeCl₃ solution using a modified method of Benzie and Strain (1996). FRAP values (expressed as mg Fe (II)/g of the sample) for the bioactive compounds were then extrapolated from the standard curve.

**Determination of iron (Fe²⁺) chelating ability**

The Fe²⁺ chelating ability of the bioactive compounds (catechin, caffeine and theobromine) was determined using a modified method of Minotti and Aust (1987). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe²⁺ chelating ability of the compounds was subsequently calculated.
Determinant of nitric oxide (NO) radical scavenging ability

Nitric oxide (NO) generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci et al., 1994. The nitrite generated in the presence or absence of the bioactive compounds were estimated using a standard curve based on sodium nitrite solutions of known concentrations.

Lipid peroxidation assay

Preparation of brain homogenates: The rats were sacrificed after exposure to mild diethyl ether anaesthetic conditions and the whole brain tissue was quickly isolated and placed on ice and then weighed. The tissue was subsequently homogenized and were centrifuged for 10 min at 3000 rpm to yield a low-speed supernatant (S1) fraction kept for lipid peroxidation assay (Belle et al., 2004).

The lipid peroxidation assay was carried out using the modified method (Ohkawa et al., 1979). Briefly 100 µL of tissue homogenate S1 was mixed with a reaction mixture containing 30 µL of 0.1 M Tris-HCl buffer (pH 7.4), sample extract (0-100 µL) and 30 µL of 250 µM freshly prepared FeSO₄ (the procedure was also carried out using 5 mM Sodium nitroprusside and 15 mM cisplatin as prooxidants). The volume was made up to 300 µL by water before incubation at 37°C for 2 hours. The colour reaction was developed by adding 300 µL 8.1% SDS (Sodium dodecyl sulphate) to the reaction mixture containing S1, this was followed by the addition of 500 µL of acetic acid/HCl (pH 3.4) mixture and 500 µL 0.8% TBA (Thiobarbituric Acid). The test tubes were incubated at 100°C for 1 hour. TBARS (Thiobarbituric Acid Reactive Substances) produced were measured at 532nm in the JENWAY UV-Visible spectrophotometer and the absorbance was compared with the standard curve using MDA (Malondialdehyde).

Enzyme inhibition assay

Cholinesterases activity assay: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) inhibitory activities were determined according to the method of Ellman et al., (1961). 0.05mM acetylthiocholine iodide (100µL) was added as the substrate, and AChE activity was determined by UV-Visible spectrophotometer from the absorbance changes at 412 nm for 3 min at 25°C while 100 µL of 0.05 mM Butyrylthiocholine iodide was used as a substrate to assay butyrylcholinesterase (BuChE) enzyme activity while all the other reagents and conditions were the same. The AChE and BuChE inhibitory activities were expressed as percentage inhibition.

RESULTS

These bioactive compounds have high antioxidant potentials. The values of antioxidant potential of the studied bioactive compounds expressed as the percentage of the inhibition of the DPPH radical is presented in Table 1. Among the bioactive compounds, Catechin possessed the highest activity. The IC₅₀ of catechin, caffeine and theobromine were 3.16±0.10, 5.16±0.12 and 3.59±0.10 µg/mL respectively. The DPPH scavenging activity of the different bioactive compounds was in the following order: Catechin>Theobromine>Caffeine. The ability of the bioactive compounds to scavenge hydroxyl radical was determined and the result is presented in Table 1. From the table, these three compounds scavenged OH radicals in a concentration dependent manner. Catechin had higher OH radical scavenging ability than theobromine followed by caffeine.

Table 1. DPPH and Hydroxyl Radical Scavenging Abilities of Catechin, Caffeine and Theobromine.

<table>
<thead>
<tr>
<th>Conc (µg/mL)</th>
<th>DPPH Catechin (% DPPH scavenged)</th>
<th>Caffeine (% DPPH scavenged)</th>
<th>Theobromine (% DPPH scavenged)</th>
<th>OH· Catechin (% OH· scavenged)</th>
<th>Caffeine (% OH· scavenged)</th>
<th>Theobromine (% OH· scavenged)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>53.25 ± 0.40¹</td>
<td>9.13 ± 0.50⁰</td>
<td>18.34 ± 0.40⁰</td>
<td>16.34 ± 0.60⁰</td>
<td>9.38 ± 0.60⁰</td>
<td>13.29 ± 0.60⁰</td>
</tr>
<tr>
<td>80</td>
<td>40.38 ± 0.50⁰</td>
<td>19.75 ± 0.48³</td>
<td>23.37 ± 0.40⁰</td>
<td>27.55 ± 0.55⁰</td>
<td>15.28 ± 0.61⁰</td>
<td>20.92 ± 0.56⁰</td>
</tr>
<tr>
<td>120</td>
<td>57.39 ± 0.50⁰</td>
<td>23.77 ± 0.60⁰</td>
<td>38.45 ± 0.60⁰</td>
<td>32.37 ± 0.64³</td>
<td>22.38 ± 0.62⁰</td>
<td>27.54 ± 0.60⁰</td>
</tr>
<tr>
<td>160</td>
<td>69.47 ± 0.50⁰</td>
<td>32.58 ± 0.50³</td>
<td>47.24 ± 0.60⁰</td>
<td>44.57 ± 0.61⁰</td>
<td>26.43 ± 0.55⁰</td>
<td>34.43 ± 0.58³</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=5). For each assay, values bearing different superscripts across the row are significantly different (p<0.05)

Among the bioactive compounds, catechin exhibited higher activity than theobromine and caffeine. The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging ability expressed in µmol TEAC/g of catechin was higher than those of theobromine and caffeine as shown in Table 2. The result of the ferric reducing antioxidant property of catechin, caffeine and theobromine were determined, expressed as ascorbic acid equivalents and presented in Table 2. The result revealed that catechin had higher reducing power than theobromine and caffeine.

Table 2. ABTS Radical Scavenging Ability and Ferric Reducing Antioxidant Property (FRAP) of Catechin, Caffeine and Theobromine.

<table>
<thead>
<tr>
<th>Bioactive Compounds</th>
<th>ABTS (µmol TEAC/g)</th>
<th>FRAP (mg Fe (II)/g of the sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>6.85 ± 0.55⁰</td>
<td>84.59 ± 0.20⁰</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.57 ± 0.40⁰</td>
<td>15.26 ± 0.29⁰</td>
</tr>
<tr>
<td>Theobromine</td>
<td>2.78 ± 0.60⁰</td>
<td>34.39 ± 0.30⁰</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=5). For each assay, values bearing different superscripts down the column are significantly different (p<0.05).
Catechin had a higher iron chelating and nitric oxide scavenging abilities compared to the other two bioactive compounds. The result of the iron chelating ability of the bioactive compounds revealed the following: Catechin>Theobromine>Caffeine and presented in Table 3.

Also, the nitric oxide scavenging ability showed that catechin had the best % inhibition amongst the three, followed by theobromine and caffeine as presented in table 3. Incubation of rats’ brain homogenate with 250mM Fe2SO4 resulted in a significant (P<0.05) increase in brain malondialdehyde (MDA) content as shown in Table 4. However, the introduction of the bioactive compounds caused a significant dose-dependent decrease (P < 0.05) in MDA content of the Fe2+-stressed brain homogenate. Nevertheless, catechin had a higher inhibitory effect on MDA production in the brain than theobromine followed by caffeine. In a similar manner, the ability of the compounds to inhibit sodium nitroprusside (SNP) induced lipid peroxidation and cisplatin induced lipid peroxidation were assessed and presented in table 4. Incubation of brain tissue with cisplatin resulted in an increase in brain MDA content. Catechin, caffeine and theobromine were also able to lower the brain MDA content in dose dependent manner with catechin showing a higher inhibitory effect than theobromine and caffeine.

Furthermore, the cholinesterases inhibitory effect of the extracts was assessed and the results are presented in Figures 1-2 respectively. Catechin, caffeine and theobromine inhibited acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in a dose-dependent manner with theobromine showing stronger inhibition on AChE and BChE activities than the others.

<table>
<thead>
<tr>
<th>Table 3. Iron Chelating and Nitric Oxide Scavenging Abilities of Catechin, Caffeine and Theobromine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc(µg/mL)</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>80</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>160</td>
</tr>
</tbody>
</table>

Values are expressed as Mean_± SEM (n=3). For each assay, values bearing different superscripts across the row are significantly different (p<0.05)
DISCUSSION

The scavenging activities of caffeine, catechin and theobromine were determined using free radicals of DPPH and ABTS radical scavenging abilities (Tables 1 and 2) which showed that the bioactive compounds can prevent radical-induced oxidative damage. This study showed that catechin possessed the highest antioxidant activity as compared to other bioactive compounds while caffeine had the lowest scavenging effects. This is positively correlated with the phenolic content in catechin. Accordingly, several reports have indicated that phenolic compounds such as catechin have higher antioxidant capacity than methylxanthines (Piluzza and Bullitta, 2011; Saeed et al., 2012; Katada et al., 2020) and the possible mechanism behind the phenolic activity could be the redox properties of their OH groups. OH radical is one of the potent reactive oxygen species (ROS) in the living system and is produced via normal body metabolisms. OH radical acts by attacking poly-unsaturated fatty acids (PUFAs) in the cell membrane and inducing damage to the cell integrity; thus, they are regarded as detrimental reactive species in AD pathophysiological processes (Uttara et al, 2009). Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules such as protein, DNA and lipids; and causes lipid peroxidation (Uttara et al, 2009). Methylxanthines can exhibit pro-oxidant properties and suppress their antioxidant capacity (Saeed et al., 2012). In this study, the OH radical produced via the reaction mixture of hydrogen peroxide (H2O2) and Fe2+ reacted with 2-deoxyribose in the reference test. However, the bioactive compounds were able to actively scavenge the OH radical and prevent the degradation of 2-deoxyribose when added to the reaction mixture in a dose-dependent manner (Table 1). The scavenging effect of catechin was higher than theobromine and caffeine. Iron chelation plays the main role for assessing antioxidant potential of an extract. The reducing power of caffeine, catechin and theobromine to reduce Fe (III) to Fe (II) ion is shown in table 3. These three bioactive compounds showed an ability to chelate Fe (II) ions in a dose-dependent manner. Sodium nitroprusside in aqueous solution at physiological pH spontaneously produces nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess’s reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Overall, catechin showed the highest nitric oxide scavenging (P<0.05) ability compared to theobromine and caffeine (Table 3).

The antioxidant capacity of the different compounds observed in this experiment could be due to the presence of high phenolics compounds. Catechin is more potent compared to other bioactive compounds used and this agrees with previous reports (Gorinstein et al., 2005; Maisuthisakul et al., 2017 Katada et al., 2020) which have shown that polyphenols increase the antioxidant activity. Evidence has suggested that transition metals, in particular Fe, are potent catalysts of free radicals (Oboh et al., 2012). Aggregation of Fe in the brain with respect to age and disease causes oxidative damage. The body requires a little quantity of Fe in the metabolism of some proteins such as hemoglobin, myoglobin, xanthine oxidase, and other Fe proteins, yet the free form of Fe in the cytosol and mitochondria can cause considerable oxidative damage by increasing superoxide production (Oboh et al., 2012) via Fenton reaction, where Fe2+ is stoichiometrically oxidized by H2O2 to Fe3+, producing OH radical and inducing lipid peroxidation (Uttara et al., 2009). The Fe2+ chelating ability of the compounds, which follows a dose–dependent manner and is expressed as percentage, can chelate radical-induced metals in the brain cells. The highest percentage of Fe2+ chelation was achieved with the highest doses of the three compounds, which could also be due to their phenolic content. One of the consequences of uncontrolled oxidative stress is injury to cells, tissues, and organs caused by oxidative damage and this can inflict direct damage to lipids. Incubation of Fe2+, sodium nitroprusside and cisplatin with brain homogenates caused a significant (p < 0.05) increase in thiobarbituric acid reactive substances (TBARS) level, indicating oxidative damage induced by free radicals generated by Fe2+, SNP and cisplatin. Sodium nitroprusside, an antihypertensive drug performs its function through the relaxation of vascular smooth muscle; resulting to its dilation of peripheral arteries and veins. However, earlier studies have shown that photo degradation of SNP ultimately produces NO, [(CN)5–Fe]3+ and [(CN)4–Fe]2+ species (Bates et al., 1990). Nitric oxide is an essential compound involved in many aspects of health including the pathophysiology of disorders such as Alzheimer’s and Parkinson’s diseases, trauma and seizures (PolPicón-Pagés et al,2019). The protection offered by catechin confirms its ability to suppress NO production and indicates its therapeutic use in the accidental toxicities resulting from the potential overload of iron and SNP (Chen et al., 2012). The free form of Fe has been linked to the etiology of neurodegenerative conditions, owing to the ability of Fe to cross the blood brain barrier in conditions of extracellular Fe overload and its involvement in peroxyl and alkoxyl radical formation, which can initiate lipid oxidation via the Fenton reaction (Volinsky and Kinnunen, 2013). However, the bioactive compounds were able to reduce the TBARS level in both Fe2+, SNP and cisplatin-induced lipid peroxidation especially catechin. Inhibition of TBARS production revealed by catechin could be due to the phenolic content, as phenolics have been shown to prevent lipid peroxidation or TBARS production (Mushtaq and Wani, 2013). Similarly, catechin has been found to decrease production of malondialdehyde activity (MDA) suggesting a protective role of catechin from peroxidative stress and prevention of platelet aggregation (Chen et al., 2012; Yan et al., 2020).

Inhibition of AChE and BChE activities prevent the breakdown of acetylcholine, thereby increasing communication between nerve cells (Odubanjo et al., 2013; Kim et al., 2021) Caffeine, catechin and theobromine were
able to inhibit AChE and BChE activities, with theobromine showing the best inhibition while catechin was the least. This confirms previous studies that micromolar concentration of methylxanthines would be expected to cause only a modest blockage of adenosine receptors and also alter cholinergic receptors. Caffeine through blockade of adenosine receptors would be expected to indirectly influence the function of most neuronal pathways in the brain, cholinergic system inclusive (dePaula and Farah, 2019). Many studies have also reported that coffee consumption improves cognitive performance and this may be primarily due to caffeine content (Eskelinen et al., 2009; Kim et al., 2021). Catechin appearing to be the least cholinesterase inhibitor in our study supports previous studies where no association between tea drinking and the risk of dementia/Alzheimer’s disease was found (dePaula and Farah, 2019). This could be due to lesser caffeine content in tea or the fact that other components than caffeine in coffee confers the protective effect (dePaula and Farah, 2019; Eskelinen et al., 2009).

CONCLUSION
This study has presented that catechin has the highest antioxidant capacity due to the presence of flavonoids, followed by caffeine while theobromine has the least. Theobromine appeared to be the best neuroprotective agent, followed by caffeine which could be due to the ability of methylxanthines to interact with neurotransmission in different regions of the brain thereby promoting cognitive functions. All these mechanisms corroborate how these compounds manage and/or prevent neurodegeneration.

Ethical approval and consent to participate
Certificate of Research Ethical Clearance for Animal Usage with Ethical number FUTA/ETH/21/12 was issued by the Centre for Research and Development, FUTA.

AUTHORS’ CONTRIBUTIONS
Iyanuoluwa O. Ademola, Ayokunle O. Ademosun and Ganiyu Oboh prepared the manuscript. Ayokunle O. Ademosun and Ganiyu Oboh reviewed and corrected the manuscript. Ayokunle O. Ademosun and Ganiyu Oboh designed and guided the study while Iyanuoluwa O. Ademola conducted the experimental work. Data interpretation and analysis was done by Iyanuoluwa O. Ademola and Ayokunle O. Ademosun. All authors reviewed and approved the manuscript.

FINANCIAL STATEMENT
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENT
We sincerely want to acknowledge Mr Bilamin O. Popoola for taking his time to proof read this manuscript.

REFERENCES


